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(Print Name)

Date: April 23, 2007

(Signature)

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Group No.: 1632

Johannes Auer, et al.

Serial No.: 10/591,045

Filed: August 29, 2006

For: METHOD FOR THE RECOMBINANT EXPRESSION OF AN N-TERMINAL FRAGMENT OF  
HEPATOCYTE GROWTH FACTOR

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Attached please find the certified copy of the foreign application from which priority is claimed for this case:

<u>Country</u>	<u>Application No.</u>	<u>Filing Date</u>
Europe	04004951.2	March 3, 2004

Respectfully submitted,

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The attached documents  
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European patent application  
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**Patentanmeldung Nr.    Patent application No.    Demande de brevet n°**

04004951.2

Der Präsident des Europäischen Patentamts;  
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets  
p.o.

**R C van Dijk**



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Application no.: 04004951.2  
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SUISSE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:  
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.  
If no title is shown please refer to the description.  
Si aucun titre n'est indiqué se référer à la description.)

Method for the recombinant expression of an N-terminal fragment of hepatocyte growth factor

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s)  
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PL PT RO SE SI SK TR LI

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2008 EP-SR

**Method for the recombinant expression of an N-terminal fragment  
of hepatocyte growth factor**

The invention relates to a method for the recombinant expression of a N-terminal four kringle-containing fragment of hepatocyte growth factor.

**Background of the Invention**

5 Hepatocyte growth factor (HGF/SF) is a polypeptide identified and purified by Nakamura, T., et al., Biochem. Biophys. Res. Commun. 22 (1984) 1450-1459. It was further found that hepatocyte growth factor is identical to scatter factor (SF), Weidner, K.M., et al., Proc. Natl. Acad. Sci. USA 88 (1991) 7001-7005. HGF is a glycoprotein involved in the development of a number of cellular phenotypes  
10 including proliferation, mitogenesis, formation of branching tubules and, in the case of tumor cells, invasion and metastasis. For a status review, see Stuart, K.A., et al., Int. J. Exp. Pathol. 81 (2000) 17-30.

Both rat HGF and human HGF have been sequenced and cloned (Miyazawa, K. et al., Biochem. Biophys. Res. Comm. 163 (1989) 967-973; Nakamura, T., et al.,  
15 Nature 342 (1989) 440-443; Seki, T., et al., Biochem. and Biophys. Res. Comm. 172 (1990) 321-327; Tashiro, K., et al., Proc. Natl. Acad. Sci. USA 87 (1990) 3200-3204; Okajima, A., et al., Eur. J. Biochem. 193 (1990) 375-381).

HGF is a protein with high similarity to human plasminogen (38% amino acid sequence identity). HGF and plasminogen are both synthesized as a single chain  
20 polypeptide which is proteolytically processed to a disulfide-linked heterodimer. HGF contains an N-terminal domain four consecutive kringle domains and a carboxyterminal protease-like domain. Different truncated HGF variants have been described. NK1 is the shortest HGF variant described. NK1 contains amino acids 32-210 and is truncated after the first kringle domain (Lokker, N.A., and Godowski,  
25 P.J., J. Biol. Chem. 268 (1993) 17145-17150). NK2 consists of the N-terminal amino acid terminus and kringle 1 and kringle 2 and is the naturally occurring product of an alternatively spliced HGF mRNA (Chan, A.M., et al., Science 254 (1991) 1382-1385). Further HGF variants containing parts of the heavy chain of HGF (amino acids 1-494, containing the alpha-subunit of HGF from amino acids 1-463) are  
30 described by Lokker, N.A., EMBO J. 11 (1992) 2503-2510).

It was further found that an HGF/SF fragment, termed NK4, consisting of the N-terminal hairpin domain and the four kringle domains of HGF/SF has pharmacological properties that are completely different from those of HGF/SF, and is an antagonist to the influence of HGF/SF on the motility and the invasion of colon cancer cells, and is, in addition, an angiogenesis inhibitor that suppresses tumor growth and metastasis (Parr, C., et al., Int. J. Cancer 85 (2000) 563-570; Kuba, K., et al., Cancer Res. 60 (2000) 6737-6743; Date, K., et al., FEBS Lett. 420 (1997) 1-6; Date, K., et al., Oncogene 17 (1989) 3045-3054).

NK4 is prepared according to the state of the art (Date, K., et al., FEBS Lett. 420 (1997) 1-6) by recombinant expression of HGF cDNA in CHO cells and subsequent digestion with pancreatic elastase. Two other isoforms of HGF (NK1 and NK2) encoding the N-terminal domain and kringle 1, and the N-terminal domain and kringles 1 and 2, respectively, were produced in E.coli (Stahl, S.J., Biochem. J. 326 (1997) 763-772). However, this method results only in about an amount of HGF-derived proteins which is about 10-20% of the total protein.

#### Summary of the Invention

The invention provides a method for the production of the alpha-chain of HGF or a fragment thereof (NK polypeptide) by expression of a nucleic acid encoding said NK polypeptide in a microbial host cell, isolation of inclusion bodies containing said NK polypeptide in denatured form, solubilization of the inclusion bodies and naturation of the denatured NK polypeptide, characterized in that in said nucleic acid at least one of the codons of amino acids selected from the group consisting of codons at positions 33, 35 and 36 is CGT.

Amino acid (aa) and codon numbering is according to the sequence shown in Swiss-Prot P14210, wherein aa (amino acid) 1-31 denotes signal sequence, aa 32-494 denotes alpha chain, aa 128-206 kringle 1, aa 211-288 kringle 2, aa 305-383 kringle 3 and aa391-469 kringle 4.

Surprisingly it was found, that modification of at least one of the codons of the DNA sequence of positions 33, 35 and 36 (codon 33 and 36 encode arginine, numbering according to M73239) results in an increase of the expression yield of about 100% or more. It is further preferred that the codon for amino acid 32 is

changed from encoding Gln to encoding Ser in order to improve splitting off N-terminal methionine.

5 NK polypeptides according to the invention consist of aa 32-494 or a N-terminal fragment thereof (always beginning with aa32), preferably fragment aa 32-478, the smallest fragment being aa 32-207. All NK polypeptides according to the invention show activity in a scatter assay according to Example 4.

10 The invention further provides a nucleic acid encoding an NK polypeptide consisting of aa 32-494 or an N-terminal fragment thereof, beginning with aa 32, preferably fragments aa 32-x, wherein x is a number between 207 and 478, and x is preferably 207 or 478, characterized in that at least one of the codons of amino acids selected from the group consisting of codons at positions 33, 35 and 36 is CGT. Preferably, all codons at positions 33, 35 and 36 are CGT.

15 In a preferred embodiment of the invention aa 32 is changed from glutamine to serine to improve homogeneity of the protein ( cleavage of N-terminal methionine).

It is further preferred to introduce two translational stop codons (TAA, TAG and/or and TGA) at the end of the nucleic acid encoding the NK polypeptide in order to stop the translation at a position equivalent to the end of desired polypeptide.

## 20 Detailed Description of the Invention

Human HGF is a disulfide-linked heterodimer, which can be cleaved in an  $\alpha$ -subunit of 463 amino acids and a  $\beta$ -subunit of 234 amino acids, by cleavage between amino acids R494 and V495. The N-terminus of the  $\alpha$ -chain is preceded by 31 amino acids started with a methionine group. This segment includes a signal sequence of 31 amino acids. The  $\alpha$ -chain starts at amino acid 32 and contains four kringle domains. The so-called "hairpin domain" consists of amino acids 70-96. The kringle 1 domain consists of amino acids 128-206. The kringle 2 domain consists of amino acids 211-288, the kringle 3 domain consists of amino acids 305-383, and the kringle 4 domain consists of amino acids 391-469 of the  $\alpha$ -chain, approximately. There exist variations of these sequences, essentially not affecting the biological

properties of NK polypeptides (especially not affecting its activities antagonistic to HGF and its antiangiogenic activities), which variations are described, for example, in WO 93/23541. Also the length of NK polypeptides can vary within a few amino acids as long as its biological properties are not affected.

5 NK1 consists of aa 32 to 206-210 of the HGF/SF $\alpha$ -chain, NK2 consists of aa32 to 288-305 and NK4 is composed of aa 32 to 447 (resp.469-494). Further NK polypeptides encoded by the nucleic acids according to the invention and which can be produced recombinantly according to the invention are described in WO 93/23541 and are e.g. 32-207, 32-303, or 32-384. NK polypeptides have the in  
10 vivo biological activity of causing inhibition of tumor growth, angiogenesis and/or metastasis.

The NK polypeptides can be produced by recombinant means in prokaryotes. For expression in prokaryotic host cells, the nucleic acid is integrated into a suitable expression vector, according to methods familiar to a person skilled in the art. Such  
15 an expression vector preferably contains a regulatable/inducible promoter. The recombinant vector is then introduced for the expression into a suitable host cell such as, e.g., E. coli and the transformed cell is cultured under conditions which allow expression of the heterologous gene. After fermentation inclusion bodies containing denatured NK polypeptide are isolated.

20 Escherichia, Salmonella, Streptomyces or Bacillus are for example suitable as prokaryotic host organisms. For the production of NK polypeptides prokaryotes are transformed in the usual manner with the vector which contains the DNA according to the invention and encoding a NK polypeptide and subsequently fermented in the usual manner. However expression yield in E. coli using the  
25 original DNA sequence of a NK polypeptide (GenBank M73239) is very low.

Inclusion bodies are found in the cytoplasm as the gene to be expressed does not contain a signal sequence. These inclusion bodies are separated from other cell components, for example by centrifugation after cell lysis.

30 The inclusion bodies were solubilized by adding a denaturing agent like 6 M guanidinium hydrochloride or 8 M urea at pH 7-9 in phosphate buffer (preferably in a concentration of 0.1 – 1.0 M, e.g.0.4 M) preferably in the presence of DTT

(Dithio-1,4-threitol). The solubilisate is diluted in phosphate buffer pH 7-9 in the presence of GSH/GSSG (preferably 2-20 mM, glutathione) and a denaturing agent in a non denaturing concentration (e.g. 2M guanidinium hydrochloride or 4 M urea) or preferably instead of guanidinium hydrochloride or urea, arginine in a concentration of about 0.3 to 1.0 M, preferably in a concentration of about 0.7M. Renaturation is performed preferably at a temperature of about 4 C and for about 48 to 160 hours.

According to the state of the art the use of Tris buffer during solubilization and renaturation leads to a considerable amount (of about 50%) of side-products which are identified by the inventors as consisting mainly of GSH-modified NK polypeptides. To the contrary, it was surprisingly found that the use of potassium phosphate buffer in a pH range between 7 and 9, preferably between pH 8 and 9, leads to a considerable improvement in yield and purity of NK polypeptides.

After renaturation is terminated the solution was dialyzed preferably against phosphate buffer pH 7-9 (preferably in a concentration of 0.1 – 1.0 M, e.g. 0.3 M) for at least 24 hours, preferably for 24 – 120 hours.

NK polypeptides can be purified after recombinant production and renaturation of the water insoluble denatured polypeptide (inclusion bodies) according to the method of the invention preferably by chromatographic methods, e.g. by affinity chromatography, hydrophobic interaction chromatography, immunoprecipitation, gel filtration, ion exchange chromatography, chromatofocussing, isoelectric focussing, selective precipitation, electrophoresis, or the like. It is preferred to purify NK polypeptides by hydrophobic interaction chromatography, preferably at pH 7-9, in the presence of phosphate buffer and/or by the use of butyl- or phenyl sepharose.

The following examples, references, figure and sequence listing are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

**Description of the Figure:**

**Figure 1:** SDS-Gel (10% NuPAGE-SDS, 5µl per lane, numbering from left to right) of NK4 protein in biomass and isolated inclusion bodies (IB).

- 5
- lane 1: standard
- lane 2: biomass
- lane 3: supernant after centrifugation
- lane 4: supernant after further centrifugation
- lane 5: IB preparation
- 10 lane 6: IB preparation after wash

**Description of the Sequences:**

15

**SEQ ID NO:1** Amino acid sequence and DNA sequence encoding the  $\alpha$ -chain of HGF, original sequence according to GenBank M73239 (without signal sequence)

**SEQ ID NO:2** Protein sequence of the  $\alpha$ -chain of HGF

**SEQ ID NO:3** Amino acid sequence and DNA sequence encoding NK4 according to the invention (amino acid sequence including N-terminal methionine, DNA sequence including two stop codons)

20 **SEQ ID NO:4** Protein sequence of NK4

**Example 1**

**Recombinant expression of NK polypeptides**

25 The NK4 polypeptide consisting of amino acid position 32 to 478 of HGF was used for cloning and recombinant expression in *Escherichia coli*. The original DNA sequence used as source of DNA was described (database identifier "gb:M73239"). PCR was performed in order to amplify and concurrently modify the DNA coding for NK4 (SEQ ID NO: 1). All methods were performed under standard conditions.

In comparison to the original DNA sequence of NK4, the following modifications were introduced:

- Elimination of the eukaryotic signal peptide sequence and fusion of the ATG start codon next to amino acid position 32 of NK4
- 5    - exchange of amino acid position 32 (position 2 in SEQ ID NO:2) from Gln to Ser in order to improve homogeneity of the protein product (Met-free)
- modification of the DNA sequence of the codons of amino acids at position 33 (AGG to CGT), 35 (AGA to CGT), and 36 (AGA to CGT) in order to improve gene expression in E.coli.
- 10   - modification of the DNA sequence of codons at position 477 (ATA to ATC) and 478 (GTC to GTT) in order to facilitate insertion of PCR product into the vector
- introduction of two translational stop codons at positions 479 (TAA) and 480 (TAG), in order to stop the translation at a position equivalent to the end of
- 15   NK4 protein domain.

The PCR-amplified DNA fragment was treated with restriction endonucleases NdeI and BanII and was ligated to the modified pQE vector (Qiagen) (elimination of His-tag as well as DHFR coding region), which was appropriately treated with NdeI and BanII. The elements of expression plasmid pQE-NK4-Ser (plasmid size 4447

20   bp) are T5 promotor/lac operator element, NK4 coding region, lambda to transcriptional termination region, *rrnB* T1 transcriptional termination region, ColE1 origin of replication and  $\beta$ -lactamase coding sequence.

The ligation reaction was used to transform E.coli competent cells, e.g. E. coli strain C600 harbouring expression helper plasmid pUBS520 (EP-0 373 365). E.coli colonies were isolated and were characterized with respect to restriction and sequence analysis of their plasmids. The selection of clones was done by analysis of the NK4 protein content after cultivation of recombinant cells in LB medium in the presence of appropriate antibiotics and after induction of the gene expression by

25   addition of IPTG (1mM). The protein pattern of cell lysates were compared by PAGE. The recombinant E.coli clone showing the highest proportion of NK4 protein was selected for the production process. Fermentation was performed

30   under standard conditions and inclusion bodies were isolated. Yield: 130 g/l net weight of cells with 30%-40% NK4 of total protein.

NK1 and NK2 can be produced recombinantly in an analogous manner.

### Example 2

#### **Solubilization and naturation**

5 Inclusion bodies were dissolved over night in a buffer containing 6 M guanidinium hydrochloride, 0.1 M potassium phosphate pH 8.5 (by titration with 10 M KOH), 1 mM EDTA, 0.01 mM DTT. The concentration of the dissolved protein was determined by Biuret assay and finally adjusted to a concentration of 25 mg total protein/ml at room temperature.

10 This NK-solubilise was diluted to a concentration of 0.4 mg/ml in a buffer containing 0.7 M arginine, 0.1 M potassium phosphate pH 8.5 (by titration with conc. HCl), 10 mM GSH, 5 mM GSSG and 1 mM EDTA. This renaturation assay was incubated between 2 and 8 days at 4°C. After obtaining the maximal renaturation efficacy, the renaturation assay of 15 l volume was concentrated to 3 l using a tangential flow filtration unit (MW cut off: 10 kDa, Sartorius). It was  
15 subsequently dialyzed against 3 times 50 l buffer containing 0.3 M potassium phosphate at pH 8.0 for at least 3 x 24 hours, optimally for 5 days in total.

### Example 3

#### **Purification**

Purification was performed by Heparin-Sepharose chromatography.

20 Buffer conditions:

Buffer A: 50 mM Tris pH 8.0

Buffer B: 50 mM Tris pH 8.0, 2 M NaCl

25 Gradient:            5-25%    buffer B, 2 column volumes  
                         25-55%    buffer B, 16 column volumes  
                         55-100% buffer B, 0.7 column volumes  
                         100%    buffer B, 2 column volumes

30 To the eluted material 1 M ammonium sulfate in 0.1 M potassium phosphate pH 8.0 was added and incubated at 4°C overnight. The sample was centrifuged and the

supernatant was loaded on a Phenyl Sepharose column (150 ml). The column was washed with 1 column volume 1 M ammonium sulfate, 50 mM potassium phosphate pH 8.0.

Elution conditions:

- 5 Buffer A: 1 M ammonium sulfate, 50 mM potassium phosphate pH 8.0  
Buffer B: 50 mM potassium phosphate pH 8.0, 40 % ethylene glycol  
0-100 % buffer B, 20 column volumes

Example 4

10 Determination of activity

a) Scatter assay

MDCK cells were subconfluently grown in tissue culture plates. Cells were treated with HGF (10 ng/ml) or with combinations of HGF and NK4. In these experiments the HGF-induced cell scattering was inhibited by the addition of a 10 to 1000-fold molar excess of NK4 at least for 90% and more, showing the functional activity.

15

b) Proliferation assay

Inhibition of the mitogenic activity of HGF by NK4 was determined by measuring DNA synthesis of adult rat hepatocytes in primary culture as described in Nakamura et al. (1989). In these experiments the HGF-induced cell proliferation was inhibited by the addition of a 10 to 1000-fold molar excess of NK4 at least for 90% and more, showing the functional activity.

20

c) Invasion assay

In this assay the invasive potential of tumor cells is analyzed. The assay was done essentially as described in Albini, A., et al., Cancer Res. 47 (1987) 3239-3245, using HT115 cells. Again, HGF-induced (10 ng/ml) cell invasion could be inhibited by a 10 to 1000-fold molar excess of NK4 at least for 90% and more, showing the functional activity.

25

**Example 5**

**Activity in vivo**

5                    Model:        Lewis Lung Carcinoma nude mouse tumor model  
                         1 x 10<sup>6</sup> Lewis Lung Carcinoma cells were s.c. implanted into male  
                         nude mice (BALB/c nu/nu).  
                         Treatment:    After 4 days, one application daily of pegylated NK4 over a period of  
                         2-4 weeks  
                         Dose:        1000 µg/mouse/day  
                                    300 µg/mouse/day  
10                               100 µg/mouse/day  
                                    placebo  
                         Result:        Treatment with NK4 shows a dose dependent suppression of  
                         primary tumor growth and metastasis, whereas no effect is seen in  
                         placebo treated groups.  
15

List of References

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- Chan, A.M., et al., Science 254 (1991) 1382-1385
- Date, K., et al., FEBS Lett. 420 (1997) 1-6
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EP 0 373 365
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- 10 Miyazawa, K. et al., Biochem. Biophys. Res. Comm. 163 (1989) 967-973
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- 15 Seki, T., et al., Biochem. and Biophys. Res. Comm. 172 (1990) 321-327
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- Stuart, K.A., et al., Int. J. Exp. Pathol. 81 (2000) 17-30
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- Weidner, K.M., et al., Proc. Natl. Acad. Sci. USA 88 (1991) 7001-7005
- 20 WO 93/23541

03. März 2004

Patent Claims

1. A nucleic acid encoding the  $\alpha$ -chain of hepatocyte growth factor or an N-terminal fragment thereof, characterized in that in said nucleic acid at least one of the codons of amino acids selected from the group consisting of codons at positions 33, 35 and 36 is CGT.  
5
2. A nucleic acid according to claim 1, characterized in that the codons of amino acids at positions 33, 35 and 36 are CGT.
3. Method for the production of  $\alpha$ -chain of hepatocyte growth factor or an N-terminal fragment thereof (NK polypeptide) by expression of a nucleic acid encoding said NK polypeptide in a microbial host cell, isolating of inclusion bodies containing said NK polypeptide in denatured form, solubilization of the inclusion bodies and naturation of the denatured NK polypeptide, characterized in that in said nucleic acid at least one of the codons of amino acids selected from the group consisting of codons at positions 33, 35 and 36 is CGT.  
10  
15
4. Method according to claim 3, characterized in that the codons of amino acids at positions 33, 35 and 36 are CGT.

Abstract

5 A method for the production of  $\alpha$ -chain of hepatocyte growth factor or an N-terminal fragment thereof (NK polypeptide) by expression of a nucleic acid encoding said NK polypeptide in a microbial host cell, isolating of inclusion bodies containing said NK polypeptide in denatured form, solubilization of the inclusion bodies and naturation of the denatured NK polypeptide, which is characterized in that in said nucleic acid at least one of the codons of amino acids selected from the group consisting of codons at positions 33, 35 and 36 is CGT, results in an improved expression yield.

SEQUENCE LISTING

03. März 2004

<110> F. Hoffmann-La Roche AG

<120> Method for the recombinant expression of an N-terminal fragment of hepatocyte growth factor

<130> 22388

<160> 4

<170> PatentIn version 3.2

<210> 1

<211> 1389

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (1)..(1389)

<223> DNA sequence encoding the alpha-chain of hepatocyte growth factor (HGF)

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1 5 10 15	
act acc cta atc aaa ata gat cca gca ctg aag ata aaa acc aaa aaa	96
Thr Thr Leu Ile Lys Ile Asp Pro Ala Leu Lys Ile Lys Thr Lys Lys	
20 25 30	
gtg aat act gca gac caa tgt gct aat aga tgt act agg aat aaa gga	144
Val Asn Thr Ala Asp Gln Cys Ala Asn Arg Cys Thr Arg Asn Lys Gly	
35 40 45	
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Leu Pro Phe Thr Cys Lys Ala Phe Val Phe Asp Lys Ala Arg Lys Gln	
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Cys Ile Ile Gly Lys Gly Arg Ser Tyr Lys Gly Thr Val Ser Ile Thr	
100 105 110	
aag agt ggc atc aaa tgt cag ccc tgg agt tcc atg ata cca cac gaa	384
Lys Ser Gly Ile Lys Cys Gln Pro Trp Ser Ser Met Ile Pro His Glu	
115 120 125	

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225					230					235					240	
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Tyr	Thr	Leu	Asp	Pro	His	Thr	Arg	Trp	Glu	Tyr	Cys	Ala	Ile	Lys	Thr	
				245					250					255		
tgc	gct	gac	aat	act	atg	aat	gac	act	gat	gtt	cct	ttg	gaa	aca	act	816
Cys	Ala	Asp	Asn	Thr	Met	Asn	Asp	Thr	Asp	Val	Pro	Leu	Glu	Thr	Thr	
			260					265					270			
gaa	tgc	atc	caa	ggt	caa	gga	gaa	ggc	tac	agg	ggc	act	gtc	aat	acc	864
Glu	Cys	Ile	Gln	Gly	Gln	Gly	Glu	Gly	Tyr	Arg	Gly	Thr	Val	Asn	Thr	
		275					280					285				
att	tgg	aat	gga	att	cca	tgt	cag	cgt	tgg	gat	tct	cag	tat	cct	cac	912
Ile	Trp	Asn	Gly	Ile	Pro	Cys	Gln	Arg	Trp	Asp	Ser	Gln	Tyr	Pro	His	
		290				295					300					
gag	cat	gac	atg	act	cct	gaa	aat	ttc	aag	tgc	aag	gac	cta	cga	gaa	960
Glu	His	Asp	Met	Thr	Pro	Glu	Asn	Phe	Lys	Cys	Lys	Asp	Leu	Arg	Glu	
305					310					315					320	
aat	tac	tgc	cga	aat	cca	gat	ggg	tct	gaa	tca	ccc	tgg	tgt	ttt	acc	1008
Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Gly	Ser	Glu	Ser	Pro	Trp	Cys	Phe	Thr	
				325				330						335		
act	gat	cca	aac	atc	cga	gtt	ggc	tac	tgc	tcc	caa	att	cca	aac	tgt	1056
Thr	Asp	Pro	Asn	Ile	Arg	Val	Gly	Tyr	Cys	Ser	Gln	Ile	Pro	Asn	Cys	
			340					345					350			

gat atg tca cat gga caa gat tgt tat cgt ggg aat ggc aaa aat tat	1104
Asp Met Ser His Gly Gln Asp Cys Tyr Arg Gly Asn Gly Lys Asn Tyr	
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atg ggc aac tta tcc caa aca aga tct gga cta aca tgt tca atg tgg	1152
Met Gly Asn Leu Ser Gln Thr Arg Ser Gly Leu Thr Cys Ser Met Trp	
370 375 380	
gac aag aac atg gaa gac tta cat cgt cat atc ttc tgg gaa cca gat	1200
Asp Lys Asn Met Glu Asp Leu His Arg His Ile Phe Trp Glu Pro Asp	
385 390 395 400	
gca agt aag ctg aat gag aat tac tgc cga aat cca gat gat gat gct	1248
Ala Ser Lys Leu Asn Glu Asn Tyr Cys Arg Asn Pro Asp Asp Asp Ala	
405 410 415	
cat gga ccc tgg tgc tac acg gga aat cca ctc att cct tgg gat tat	1296
His Gly Pro Trp Cys Tyr Thr Gly Asn Pro Leu Ile Pro Trp Asp Tyr	
420 425 430	
tgc cct att tct cgt tgt gaa ggt gat acc aca cct aca ata gtc aat	1344
Cys Pro Ile Ser Arg Cys Glu Gly Asp Thr Thr Pro Thr Ile Val Asn	
435 440 445	
tta gac cat ccc gta ata tct tgt gcc aaa acg aaa caa ttg cga	1389
Leu Asp His Pro Val Ile Ser Cys Ala Lys Thr Lys Gln Leu Arg	
450 455 460	

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<400> 2

Gln Arg Lys Arg Arg Asn Thr Ile His Glu Phe Lys Lys Ser Ala Lys	
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Val Asn Thr Ala Asp Gln Cys Ala Asn Arg Cys Thr Arg Asn Lys Gly	
35 40 45	
Leu Pro Phe Thr Cys Lys Ala Phe Val Phe Asp Lys Ala Arg Lys Gln	
50 55 60	
Cys Leu Trp Phe Pro Phe Asn Ser Met Ser Ser Gly Val Lys Lys Glu	
65 70 75 80	

Phe Gly His Glu Phe Asp Leu Tyr Glu Asn Lys Asp Tyr Ile Arg Asn  
85 90 95

Cys Ile Ile Gly Lys Gly Arg Ser Tyr Lys Gly Thr Val Ser Ile Thr  
100 105 110

Lys Ser Gly Ile Lys Cys Gln Pro Trp Ser Ser Met Ile Pro His Glu  
115 120 125

His Ser Phe Leu Pro Ser Ser Tyr Arg Gly Lys Asp Leu Gln Glu Asn  
130 135 140

Tyr Cys Arg Asn Pro Arg Gly Glu Glu Gly Gly Pro Trp Cys Phe Thr  
145 150 155 160

Ser Asn Pro Glu Val Arg Tyr Glu Val Cys Asp Ile Pro Gln Cys Ser  
165 170 175

Glu Val Glu Cys Met Thr Cys Asn Gly Glu Ser Tyr Arg Gly Leu Met  
180 185 190

Asp His Thr Glu Ser Gly Lys Ile Cys Gln Arg Trp Asp His Gln Thr  
195 200 205

Pro His Arg His Lys Phe Leu Pro Glu Arg Tyr Pro Asp Lys Gly Phe  
210 215 220

Asp Asp Asn Tyr Cys Arg Asn Pro Asp Gly Gln Pro Arg Pro Trp Cys  
225 230 235 240

Tyr Thr Leu Asp Pro His Thr Arg Trp Glu Tyr Cys Ala Ile Lys Thr  
245 250 255

Cys Ala Asp Asn Thr Met Asn Asp Thr Asp Val Pro Leu Glu Thr Thr  
260 265 270

Glu Cys Ile Gln Gly Gln Gly Glu Gly Tyr Arg Gly Thr Val Asn Thr  
275 280 285

Ile Trp Asn Gly Ile Pro Cys Gln Arg Trp Asp Ser Gln Tyr Pro His  
290 295 300

Glu His Asp Met Thr Pro Glu Asn Phe Lys Cys Lys Asp Leu Arg Glu  
305 310 315 320

Asn Tyr Cys Arg Asn Pro Asp Gly Ser Glu Ser Pro Trp Cys Phe Thr  
325 330 335

Thr Asp Pro Asn Ile Arg Val Gly Tyr Cys Ser Gln Ile Pro Asn Cys  
340 345 350

Asp Met Ser His Gly Gln Asp Cys Tyr Arg Gly Asn Gly Lys Asn Tyr  
355 360 365

Met Gly Asn Leu Ser Gln Thr Arg Ser Gly Leu Thr Cys Ser Met Trp  
370 375 380

Asp Lys Asn Met Glu Asp Leu His Arg His Ile Phe Trp Glu Pro Asp  
385 390 395 400

Ala Ser Lys Leu Asn Glu Asn Tyr Cys Arg Asn Pro Asp Asp Ala  
405 410 415

His Gly Pro Trp Cys Tyr Thr Gly Asn Pro Leu Ile Pro Trp Asp Tyr  
420 425 430

Cys Pro Ile Ser Arg Cys Glu Gly Asp Thr Thr Pro Thr Ile Val Asn  
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Leu Asp His Pro Val Ile Ser Cys Ala Lys Thr Lys Gln Leu Arg  
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aag act acc cta atc aaa ata gat cca gca ctg aag ata aaa acc aaa Lys Thr Thr Leu Ile Lys Ile Asp Pro Ala Leu Lys Ile Lys Thr Lys 20 25 30	96
aaa gtg aat act gca gac caa tgt gct aat aga tgt act agg aat aaa Lys Val Asn Thr Ala Asp Gln Cys Ala Asn Arg Cys Thr Arg Asn Lys 35 40 45	144
gga ctt cca ttc act tgc aag gct ttt gtt ttt gat aaa gca aga aaa Gly Leu Pro Phe Thr Cys Lys Ala Phe Val Phe Asp Lys Ala Arg Lys 50 55 60	192
caa tgc ctc tgg ttc ccc ttc aat agc atg tca agt gga gtg aaa aaa Gln Cys Leu Trp Phe Pro Phe Asn Ser Met Ser Ser Gly Val Lys Lys 65 70 75 80	240
gaa ttt ggc cat gaa ttt gac ctc tat gaa aac aaa gac tac att aga Glu Phe Gly His Glu Phe Asp Leu Tyr Glu Asn Lys Asp Tyr Ile Arg 85 90 95	288
aac tgc atc att ggt aaa gga cgc agc tac aag gga aca gta tct atc Asn Cys Ile Ile Gly Lys Gly Arg Ser Tyr Lys Gly Thr Val Ser Ile 100 105 110	336
act aag agt ggc atc aaa tgt cag ccc tgg agt tcc atg ata cca cac Thr Lys Ser Gly Ile Lys Cys Gln Pro Trp Ser Ser Met Ile Pro His 115 120 125	384
gaa cac agc ttt ttg cct tgc agc tat cgg ggt aaa gac cta cag gaa Glu His Ser Phe Leu Pro Ser Ser Tyr Arg Gly Lys Asp Leu Gln Glu 130 135 140	432
aac tac tgt cga aat cct cga ggg gaa gaa ggg gga ccc tgg tgt ttc Asn Tyr Cys Arg Asn Pro Arg Gly Glu Glu Gly Gly Pro Trp Cys Phe 145 150 155 160	480
aca agc aat cca gag gta cgc tac gaa gtc tgt gac att cct cag tgt Thr Ser Asn Pro Glu Val Arg Tyr Glu Val Cys Asp Ile Pro Gln Cys 165 170 175	528
tca gaa gtt gaa tgc atg acc tgc aat ggg gag agt tat cga ggt ctc Ser Glu Val Glu Cys Met Thr Cys Asn Gly Glu Ser Tyr Arg Gly Leu 180 185 190	576
atg gat cat aca gaa tca ggc aag att tgt cag cgc tgg gat cat cag Met Asp His Thr Glu Ser Gly Lys Ile Cys Gln Arg Trp Asp His Gln 195 200 205	624
aca cca cac cgg cac aaa ttc ttg cct gaa aga tat ccc gac aag ggc Thr Pro His Arg His Lys Phe Leu Pro Glu Arg Tyr Pro Asp Lys Gly 210 215 220	672
ttt gat gat aat tat tgc cgc aat ccc gat ggc cag ccg agg cca tgg Phe Asp Asp Asn Tyr Cys Arg Asn Pro Asp Gly Gln Pro Arg Pro Trp 225 230 235 240	720

tgc tat act ctt gac cct cac acc cgc tgg gag tac tgt gca att aaa	768
Cys Tyr Thr Leu Asp Pro His Thr Arg Trp Glu Tyr Cys Ala Ile Lys	
245 250 255	
aca tgc gct gac aat act atg aat gac act gat gtt cct ttg gaa aca	816
Thr Cys Ala Asp Asn Thr Met Asn Asp Thr Asp Val Pro Leu Glu Thr	
260 265 270	
act gaa tgc atc caa ggt caa gga gaa ggc tac agg ggc act gtc aat	864
Thr Glu Cys Ile Gln Gly Gln Gly Glu Gly Tyr Arg Gly Thr Val Asn	
275 280 285	
acc att tgg aat gga att cca tgt cag cgt tgg gat tct cag tat cct	912
Thr Ile Trp Asn Gly Ile Pro Cys Gln Arg Trp Asp Ser Gln Tyr Pro	
290 295 300	
cac gag cat gac atg act cct gaa aat ttc aag tgc aag gac cta cga	960
His Glu His Asp Met Thr Pro Glu Asn Phe Lys Cys Lys Asp Leu Arg	
305 310 315 320	
gaa aat tac tgc cga aat cca gat ggg tct gaa tca ccc tgg tgt ttt	1008
Glu Asn Tyr Cys Arg Asn Pro Asp Gly Ser Glu Ser Pro Trp Cys Phe	
325 330 335	
acc act gat cca aac atc cga gtt ggc tac tgc tcc caa att cca aac	1056
Thr Thr Asp Pro Asn Ile Arg Val Gly Tyr Cys Ser Gln Ile Pro Asn	
340 345 350	
tgt gat atg tca cat gga caa gat tgt tat cgt ggg aat ggc aaa aat	1104
Cys Asp Met Ser His Gly Gln Asp Cys Tyr Arg Gly Asn Gly Lys Asn	
355 360 365	
tat atg ggc aac tta tcc caa aca aga tct gga cta aca tgt tca atg	1152
Tyr Met Gly Asn Leu Ser Gln Thr Arg Ser Gly Leu Thr Cys Ser Met	
370 375 380	
tgg gac aag aac atg gaa gac tta cat cgt cat atc ttc tgg gaa cca	1200
Trp Asp Lys Asn Met Glu Asp Leu His Arg His Ile Phe Trp Glu Pro	
385 390 395 400	
gat gca agt aag ctg aat gag aat tac tgc cga aat cca gat gat gat	1248
Asp Ala Ser Lys Leu Asn Glu Asn Tyr Cys Arg Asn Pro Asp Asp Asp	
405 410 415	
gct cat gga ccc tgg tgc tac acg gga aat cca ctc att cct tgg gat	1296
Ala His Gly Pro Trp Cys Tyr Thr Gly Asn Pro Leu Ile Pro Trp Asp	
420 425 430	
tat tgc cct att tct cgt tgt gaa ggt gat acc aca cct aca atc gtt	1344
Tyr Cys Pro Ile Ser Arg Cys Glu Gly Asp Thr Thr Pro Thr Ile Val	
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<213> Artificial

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Lys Thr Thr Leu Ile Lys Ile Asp Pro Ala Leu Lys Ile Lys Thr Lys  
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Lys Val Asn Thr Ala Asp Gln Cys Ala Asn Arg Cys Thr Arg Asn Lys  
35 40 45

Gly Leu Pro Phe Thr Cys Lys Ala Phe Val Phe Asp Lys Ala Arg Lys  
50 55 60

Gln Cys Leu Trp Phe Pro Phe Asn Ser Met Ser Ser Gly Val Lys Lys  
65 70 75 80

Glu Phe Gly His Glu Phe Asp Leu Tyr Glu Asn Lys Asp Tyr Ile Arg  
85 90 95

Asn Cys Ile Ile Gly Lys Gly Arg Ser Tyr Lys Gly Thr Val Ser Ile  
100 105 110

Thr Lys Ser Gly Ile Lys Cys Gln Pro Trp Ser Ser Met Ile Pro His  
115 120 125

Glu His Ser Phe Leu Pro Ser Ser Tyr Arg Gly Lys Asp Leu Gln Glu  
130 135 140

Asn Tyr Cys Arg Asn Pro Arg Gly Glu Glu Gly Gly Pro Trp Cys Phe  
145 150 155 160

Thr Ser Asn Pro Glu Val Arg Tyr Glu Val Cys Asp Ile Pro Gln Cys  
165 170 175

Ser Glu Val Glu Cys Met Thr Cys Asn Gly Glu Ser Tyr Arg Gly Leu  
180 185 190

Met Asp His Thr Glu Ser Gly Lys Ile Cys Gln Arg Trp Asp His Gln  
195 200 205

Thr Pro His Arg His Lys Phe Leu Pro Glu Arg Tyr Pro Asp Lys Gly  
210 215 220

Phe Asp Asp Asn Tyr Cys Arg Asn Pro Asp Gly Gln Pro Arg Pro Trp  
225 230 235 240

Cys Tyr Thr Leu Asp Pro His Thr Arg Trp Glu Tyr Cys Ala Ile Lys  
245 250 255

Thr Cys Ala Asp Asn Thr Met Asn Asp Thr Asp Val Pro Leu Glu Thr  
260 265 270

Thr Glu Cys Ile Gln Gly Gln Gly Glu Gly Tyr Arg Gly Thr Val Asn  
275 280 285

Thr Ile Trp Asn Gly Ile Pro Cys Gln Arg Trp Asp Ser Gln Tyr Pro  
290 295 300

His Glu His Asp Met Thr Pro Glu Asn Phe Lys Cys Lys Asp Leu Arg  
305 310 315 320

Glu Asn Tyr Cys Arg Asn Pro Asp Gly Ser Glu Ser Pro Trp Cys Phe  
325 330 335

Thr Thr Asp Pro Asn Ile Arg Val Gly Tyr Cys Ser Gln Ile Pro Asn  
340 345 350

Cys Asp Met Ser His Gly Gln Asp Cys Tyr Arg Gly Asn Gly Lys Asn  
355 360 365

Tyr Met Gly Asn Leu Ser Gln Thr Arg Ser Gly Leu Thr Cys Ser Met  
370 375 380

Trp Asp Lys Asn Met Glu Asp Leu His Arg His Ile Phe Trp Glu Pro  
385 390 395 400

Asp Ala Ser Lys Leu Asn Glu Asn Tyr Cys Arg Asn Pro Asp Asp Asp  
405 410 415

Ala His Gly Pro Trp Cys Tyr Thr Gly Asn Pro Leu Ile Pro Trp Asp  
420 425 430

Tyr	Cys	Pro	Ile	Ser	Arg	Cys	Glu	Gly	Asp	Thr	Thr	Pro	Thr	Ile	Val
	435						440					445			

EPO - Munich  
51  
03. März 2004

**Fig. 1**

